A Homozygous Nonsense Mutation within the Dystonin Gene Coding for the Coiled-Coil Domain of the Epithelial Isoform of BPAG1 Underlies a New Subtype of Autosomal Recessive Epidermolysis Bullosa Simplex

Richard W. Groves[1,2], Lu Liu[3], Patricia J. Dopping-Hepenstal[3], Hugh S. Markus[4], Patricia A. Lovell[3], Linda Ozoemena[3], Joey E. Lai-Cheong[1,2], Jeffrey Gawler[5], Katsushi Owaribe[6], Takashi Hashimoto[7], Jemima E. Mellerio[1,2], John B. Mee[2] and John A. McGrath[2]

Epidermolysis bullosa (EB) is a group of autosomal dominant and recessive blistering skin diseases in which pathogenic mutations have been reported in 13 different genes encoding structural proteins involved in keratinocyte integrity, as well as cell–matrix or cell–cell adhesion. We now report an inherited skin fragility disorder with a homozygous nonsense mutation in the dystonin gene (*DST*) that encodes the coiled-coil domain of the epithelial isoform of bullous pemphigoid antigen 1, BPAG1-e (also known as BP230). The mutation, p.Gln1124X, leads to the loss of hemidesmosomal inner plaques and a complete absence of skin immunostaining for BPAG1-e, as well as reduced labeling for plectin, the β4 integrin subunit, and for type XVII collagen. The 38-year-old affected individual has lifelong generalized trauma-induced spontaneous blisters and erosions, particularly around the ankles. In addition, he experiences episodic numbness in his limbs, which started at the age of 37 years. These neurological symptoms may also be due to *DST* gene mutation, although he has a concomitant diagnosis of CADASIL (cerebral arteriopathy, autosomal dominant, with subcortical infarcts and leukoencephalopathy), a cerebral small-vessel arteriopathy, which thus complicates the genotype–phenotype interpretation. With regard to skin blistering, the clinicopathological findings expand the molecular basis of EB by identifying BPAG1-e pathology in a new form of autosomal recessive EB simplex.

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INTRODUCTION

The group of inherited blistering skin diseases, known as epidermolysis bullosa (EB), represents a diverse collection of autosomal dominant and autosomal recessive disorders with varying skin, mucous membrane, and extracutaneous abnormalities (Fine *et al.*, 2008; Fine and Mellerio, 2009a, b). Originally classified into three main subtypes, namely EB simplex, junctional EB, and dystrophic EB, on the basis of the level of tissue cleavage at or close to the dermal–epidermal junction (DEJ), the latest classification of EB has been expanded to include other cell–cell or cell–matrix adhesion disorders (Fine *et al.*, 2008). Thus, the term ''EB'' now encompasses inherited disorders of hemidesmosome attachment complexes, keratin intermediate filaments, focal adhesions, and desmosome cell junctions, and involves pathogenic mutations in 13 genes encoding 11 different structural proteins (Fine *et al.*, 2008).

One skin protein that has not yet been implicated in the pathogenesis of EB, however, is bullous pemphigoid antigen 1 (BPAG1-e) (also known as BP230), a key component of hemidesmosomes and a member of the plakin family with cytoskeletal linker properties (Borradori and Sonnenberg, 1999; Leung *et al.*, 2001a; Litjens *et al.*, 2006; Sonnenberg and Liem, 2007). BPAG1-e is encoded for by the dystonin (*DST*) gene, the alternative splicing of which may give rise to

[1]St John's Institute of Dermatology, The Guy's and St Thomas' NHS Foundation Trust, St Thomas' Hospital, London, UK; [2]Division of Genetics and Molecular Medicine, King's College London, Guy's Hospital, London, UK; [3]The Robin Eady National Diagnostic Epidermolysis Bullosa Laboratory, GSTS Pathology, St Thomas' Hospital, London, UK; [4]Department of Clinical Neuroscience, St George's University of London, London, UK; [5]145 Harley Street, London, UK; [6]Division of Biological Science, Graduate School of Science, Nagoya University, Nagoya, Japan and [7]Department of Dermatology, Kurume University School of Medicine, Kurume, Fukuoka, Japan

Correspondence: John A. McGrath, Dermatology Research Laboratories, 9th Floor, Tower Wing, Guy's Hospital, Great Maze Pond, London SE1 9RT, UK. E-mail: john.mcgrath@kcl.ac.uk

Abbreviations: BPAG1, bullous pemphigoid antigen 1; CADASIL, cerebral arteriopathy, autosomal dominant, with subcortical infarcts and leukoencephalopathy; DEJ, dermal–epidermal junction; DST, dystonin; EB, epidermolysis bullosa

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multiple tissue isoforms with variable expression in the skin, neurons, muscles, and the central nervous system (Leung *et al.*, 2001b; Jefferson *et al.*, 2006; Young and Kothary, 2007, 2009). The main isoform expressed in the central nervous system is BPAG1-a, although a further neural variant, BPAG1-n, may also exist; whether BPAG1-n is expressed *in vivo*, however, is uncertain (Leung *et al.*, 2001b). In muscle, the main isoform is BPAG1-b. In the skin, there is a predominance of BPAG1-e, but some BPAG1-a (and perhaps BPAG1-n) may also be present (Sonnenberg and Liem, 2007). Details of these *DST* splice variants and their functional subdomains are illustrated in Supplementary Figure S1 online.

Mutations in the mouse *Dst* gene have previously been shown to cause sensory neuron degeneration and skin fragility (Brown *et al.*, 1995; Guo *et al.*, 1995; Pool *et al.*, 2005; Goryunov *et al.*, 2007), but no human *DST* gene pathology has been reported, apart from a t(6;15)(p11.2;p12) translocation in a 4-year-old girl that resulted in encephalopathy, severe motor and mental retardation, and delayed visual maturation (Giorda *et al.*, 2004). In that case, the chromosome 6 break point was between the BPAG1-a and BPAG1-b isoforms, but no abnormality of BPAG1-e was evident.

We now report details of an individual with a naturally occurring homozygous nonsense mutation in part of the *DST* gene that encodes for the coiled-coil domain, which is exclusively expressed in BPAG1-e and BPAG1-n isoforms.

RESULTS

Clinical features of skin blistering and neurological symptoms

The affected individual was a 38-year-old Kuwaiti man born to distantly related parents. He was the second of five siblings and the only one with a history of blisters. His two children had no skin symptoms and there was no family history of skin fragility or blistering. The patient had experienced lifelong trauma-induced spontaneous blisters and erosions, particularly around his ankles and feet (Figure 1a), although the face, trunk, and more proximal limbs were also affected. Blisters and erosions healed without delay, scarring, or milia formation. Blistering was associated with skin peeling and occasional hemorrhage, as well as hypopigmentation and some postinflammatory hyperpigmentation. Nail dystrophy of all toenails was present, particularly affecting the great toes. Hair growth was normal. There was no history of mucosal blistering, although moderate dental caries and reactive gingival inflammation were evident. The patient reported no gastrointestinal or urological symptoms.

In addition to skin blistering, the patient had a few years' history of neurological symptoms, including two episodes of collapse, recurrent bilateral headaches, and transient episodes of left arm numbness and weakness lasting a few

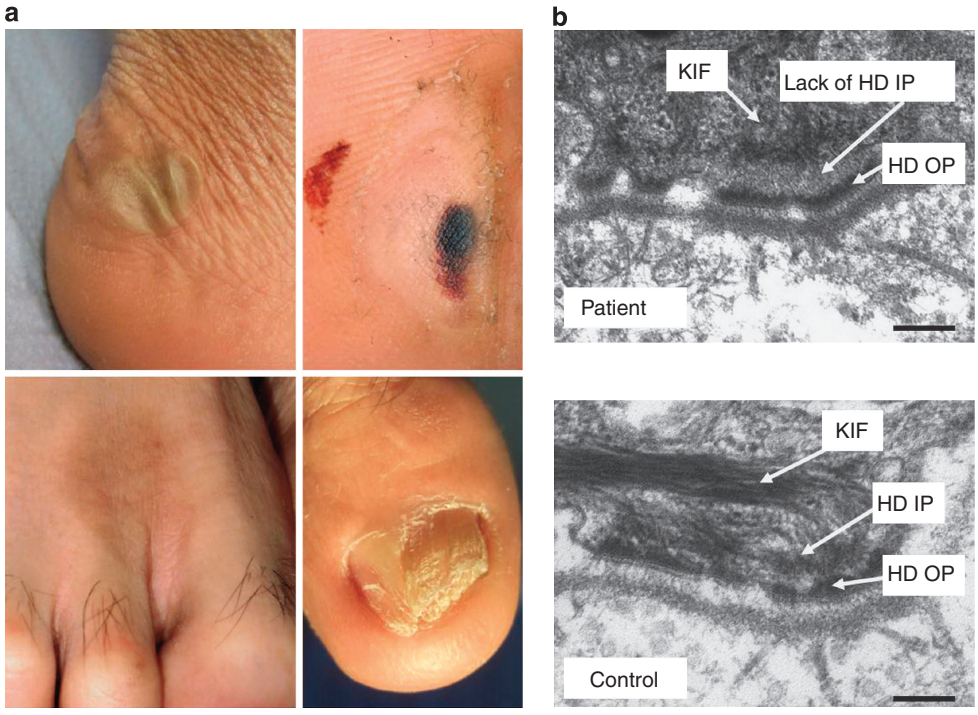

Figure 1. Ultrastructural abnormalities of hemidesmosomal inner plaques provide clues to the pathology of a new inherited skin fragility disorder. (**a**) Clinically, there is a blister measuring 2.5 × 1.5 cm on the right heel, as well as an area of earlier blistering on the right sole showing reepithelialization, some scale, minor hemorrhage, but no scarring. Other features include postinflammatory hyperpigmentation on the dorsal aspect of the right foot close to the great and second toes at the site of the old blistering, and dystrophy of the right great toe nail after previous trauma (no fungus was present on microscopy and culture). (**b**) Transmission electron microscopy of the dermal–epidermal junction (DEJ) in patient skin shows keratin intermediate filaments (KIFs) and hemidesmosomal outer plaques (HD OP), but a lack of hemidesmosomal inner plaques (HD IP). In contrast, transmission electron microscopy of the DEJ in normal control skin reveals that HDs have clearly defined HD IPs and HD OPs (bar = 100 nm).

seconds at a time. There was no history of stroke or depression. Both parents were deceased; his mother died at the age of 56 years after multiple strokes. He had two sisters and three brothers, one of whom had been given a diagnosis of ''multiple sclerosis'' after a number of neurological events that were consistent with minor strokes. Magnetic resonance brain imaging in our patient showed scattered white matter hyperintensities, but no involvement of the anterior temporal pole and the external capsule. Normal investigations included the following: intracranial and extracranial magnetic resonance angiography, cerebrospinal fluid examination including oligoclonal bands, visual brainstem, and somatosensory evoked potentials. On the basis of family history, abnormal magnetic resonance imaging, and neurological symptoms, he had a concomitant diagnosis of autosomal dominant cerebral small-vessel arteriopathy CADASIL (cerebral arteriopathy, autosomal dominant, with subcortical infarcts and leukoencephalopathy; MIM125310) (Joutel *et al.*, 1996).

Thus, our patient had a history of skin blistering resembling a form of EB; his mother, brother, and he had neurological histories consistent with CADASIL.

Transmission electron microscopy of the DEJ reveals abnormal hemidesmosomal inner plaques

The primary clue to the molecular pathology responsible for skin fragility came from ultrastructural observations and a candidate gene approach. Although no blisters or microsplits were noted at or close to the DEJ, discrete abnormalities of hemidesmosomes were evident. There were no differences from control skin in the overall number of hemidesmosomes, but the morphology of individual hemidesmosomes was abnormal. Notably, the inner plaques were poorly formed or completely absent, leading to a lucent zone between keratin filaments and the outer hemidesmosomal plaques (Figure 1b). The keratin filaments extended to where the inner plaques should be, but did not seem to associate with any plasma membrane attachment structure. The outer hemidesmosomal plaques, however, showed no gross abnormalities. Similarly, sub-basal dense plates, anchoring filaments, the lamina lucida, the lamina densa, and anchoring fibrils, were all within normal limits. Thus, transmission electron microscopy provided the specific ultrastructural clue that an abnormality of a gene encoding a structural component of the hemidesmosomal inner plaque might underlie the skin fragility in this patient. We also searched for granular osmiophilic material around vascular smooth muscle in the deep dermis, as this has been noted as a specific ultrastructural feature of CADASIL (Ishiko *et al.*, 2005), but did not identify pathognomonic changes.

Immunofluorescence microscopy and immunoblotting show an absence of BPAG1-e expression in skin/keratinocytes

Immunolabeling of the DEJ in the patient's skin showed reduced-intensity staining for several hemidesmosome-associated proteins. Notably, there was a complete absence of immunoreactvity using a BPAG1-e-specific antibody (BPC319, directed against the carboxyl terminal domain of BPAG1-e, Okumura *et al.*, 2002) compared with bright, linear labeling at the DEJ in control skin (Figure 2a). Immunoblotting using keratinocyte extracts and an additional BPAG1-e antibody (5E, directed against the carboxyl terminal domain of BPAG1-e, Hashimoto *et al.*, 1993) showed a complete absence of BPAG1-e in the patient's cells (Figure 2b). Immunostaining of skin sections from the patient also revealed markedly reduced labeling for the β4 integrin subunit at the DEJ, moderately reduced plectin immunoreactivity, and slightly diminished type XVII collagen immunolabeling (Supplementary Figure S2 online). No differences compared with control skin were noted for the α6 integrin subunit, as well as for keratin 14, laminin-332, collagen IV, or collagen VII (Supplementary Figure S2 online).

***DST* mutation screening identifies a homozygous nonsense mutation in the coiled-coil domain, present in BPAG1-e and BPAG1-n isoforms**

Sequencing of the patient's genomic DNA identified a homozygous C>T transition (c.3478C>T; GenBank NM_001723.4) that converts glutamine to a stop codon, designated p.Gln1124X (Figure 2c). This mutation occurs within the coiled-coil domain, a region that is not present in BPAG1-a or BPAG1-b (Supplementary Figure S1 online). This mutation was not identified in screening 200 ethnically matched control chromosomes. Sequencing of the *PLEC1* gene encoding plectin and the *ITGB4* gene encoding the β4 integrin subunit was also performed, but no pathogenic mutations were identified.

The nonsense mutation in *DST* does not lead to a major reduction in mRNA levels relevant to the BPAG1-e isoform

The mutation, p.Gln1124X, occurs in the penultimate exon of the transcript encoding BPAG1-e (exon 23 of 24). We assessed the consequences of the mutation in gene expression by performing real-time reverse transcriptase-PCR using RNA extracted from the patient and control skin and creating different primer pairs upstream of the mutation, spanning the mutation in the coiled-coil domain, and in the 3′ untranslated region (Figure 2d). Gene expression levels in the patient were reduced by ~25% in the 3′ untranslated region. In contrast, there was only a very slight reduction (<3%) for the other primer pairs.

***NOTCH3* mutation screening identifies a heterozygous cysteine substitution consistent with a concomitant diagnosis of CADASIL**

Sequencing of the patient's genomic DNA identified a heterozygous G>C transversion (c.1790G>C; GenBank U97669) in exon 11 of *NOTCH3* that converts cysteine to serine, designated p.Cys597Ser (not illustrated). This mutation is typical of CADASIL, in which mutations result in unpairing of a cysteine–cysteine bond in one of the epidermal growth factor-like repeats present in the extracellular portion of the transmembranous NOTCH3 protein (Joutel *et al.*, 1997). This mutation was not detected in screening 200 ethnically matched control chromosomes.

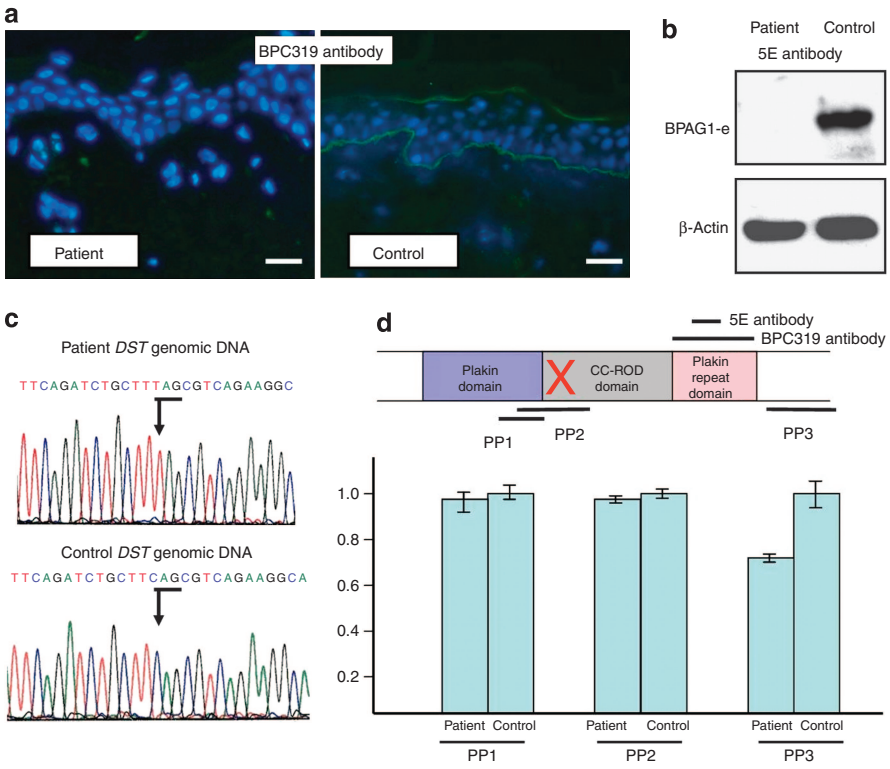

Figure 2. Loss of BPAG1-e protein expression due to a homozygous nonsense mutation in the *DST* gene. (**a**) Immunofluorescence microscopy using an antibody that recognizes the carboxyl terminal region of bullous pemphigoid antigen 1 (BPAG1)-e (clone BPC319) reveals completely undetectable immunostaining in patient skin in contrast to linear labeling at the dermal–epidermal junction (DEJ) in normal control skin. (Bar = 50 μm). (**b**) Immunoblotting using extracts from cultured keratinocytes shows a complete absence of BPAG1-e in patient material compared with control (clone 5E, carboxyl terminal region of BPAG1-e). (**c**) DNA sequencing reveals a homozygous nonsense mutation partly of the *DST* gene coding for the coiled-coil domain of BPAG1-e (and BPAG1-n). In patient genomic DNA, there is a homozygous C>T transition (c.3478C>T, GenBank NM_001723.4) that converts glutamine to a stop codon (p.Gln1124X) that is not present in wild-type sequencing. (**d**) Real-time reverse transcriptase (RT)-PCR using primer sets upstream from the mutation (PP1) and spanning the mutation in the coiled-coil domain (PP2) shows that the nonsense mutation does not lead to mRNA decay. For the primer sited in the 3′ untranslated region (UTR) (PP3), patient mRNA is reduced by only ~25%, consistent with a nonsense mutation in the penultimate exon of a gene transcript not triggering substantial mRNA decay. The schematic illustrates how RT-PCR primers relate to the domains of BPAG1-e. This schematic also illustrates the epitopes of BPAG1-e corresponding to the antibodies used for the data shown in panels **a** and **b**.

DISCUSSION

DST mutation and inherited skin fragility

Delineation of a homozygous nonsense mutation in *DST* expands the number of basement membrane genes and proteins implicated in the pathogenesis of the different forms of EB, and also highlights the importance of hemidesmosomes in maintaining epidermal integrity (Supplementary Figure S3 online). Hemidesmosomes are intricate multiprotein adhesion complexes that provide a stable attachment between the basement membrane and the stratified (and other) epithelia (Litjens *et al.*, 2006). In the skin, hemidesmosomes are composed of BPAG1-e, plectin, α6β4 integrin, tetraspanin CD151, and type XVII collagen. Together, these proteins form an intricate network that provides a structural bridge extending from keratin intermediate filaments in basal keratinocytes to the collagen fibers within the papillary dermis (Koster *et al.*, 2003). Both BPAG1-e and plectin bind to keratin filaments. For BPAG1-e, the binding to keratin occurs through its carboxyl domain, whereas its amino terminus binds to the cytoplasmic domain of type XVII collagen (Borradori and Sonnenberg, 1999; Hopkinson and Jones, 2000; Fontao *et al.*, 2003), as well as to β4 integrin and erbin (Favre *et al.*, 2001). Plectin also binds to β4 integrin (de Pereda *et al.*, 2009), and β4 integrin binds to erbin (Favre *et al.*, 2001).

The homozygous nonsense mutation in our patient led to a loss of BPAG1-e protein expression at the DEJ and in keratinocytes, as determined by immunofluorescence microscopy and western blotting using two antibodies directed against the carboxyl terminus of BPAG1-e. Nonsense mutations typically lead to mRNA decay, except when located in the last exon or the distal part of the penultimate exon (Mühlemann *et al.*, 2008; Neu-Yilik and Kulozik, 2008; Silva and Romão, 2009). The mutation p.Gln1124X is located within the proximal part of the penultimate exon but does not seem to lead to substantial mRNA decay. Nevertheless, immunostaining of the patient's skin sections showed reduced labeling for several other hemidesmosomal components that directly or indirectly associate with the amino and carboxyl domains of BPAG1-e. It is noteworthy that there was reduced protein labeling for the β4 integrin subunit, as well as for plectin and type XVII collagen. The most marked reduction in labeling was for β4 integrin, which is of interest, given recent data showing

that BPAG1-e maintains keratinocyte polarity through β4 integrin-mediated modulation of Rac1 and cofilin activation (Hamill *et al.*, 2009).

Ultrastructurally, the *DST* mutation was associated with loss of the hemidesmosomal inner plaque, but not with gross changes in the size or number of hemidesmosomal outer plaques, sub-basal dense plates, anchoring filaments, or anchoring fibrils. Keratin filaments were still seen in close apposition to the missing inner plaques, although no clear attachment structures for the filaments were evident. Although none of the skin biopsy samples obtained from the patient provided histological evidence for the level of blister formation, it is highly likely that cleavage occurred through the level of the missing inner hemidesmosomal plaques, and thus the subtype of skin fragility is probably best classified as an autosomal recessive form of EB simplex. Compared with other forms of autosomal recessive EB simplex, the severity of skin blistering in our patient was relatively mild, indicating a comparatively minor role in maintaining hemidesmosomal integrity for BPAG1-e compared with other hemidesmosome-associated proteins.

DST mutation and clinical neuropathology

In our patient, the clinical features also included some neurological abnormalities, including headaches and parasthesiae. A key question is whether these symptoms and signs are due to his *DST* gene pathology or due to a separate disorder. In support of the latter, screening of the *NOTCH3* gene identified a heterozygous missense mutation that was typical of CADASIL, an autosomal dominant small-vessel arteriopathy. In this disorder, vascular changes can be seen in the small arteries throughout the body, but clinical features are limited to the central nervous system. These include early-onset lacunar stroke, migraine usually with aura, depression, and early-onset vascular dementia. Our patient was reviewed in a UK National CADASIL clinic, however, and it was believed that these neurological symptoms were not typical of CADASIL symptomotology, suggesting perhaps that *DST* gene pathology might also be a contributing factor.

There are a number of publications linking abnormalities in the *DST* gene and BPAG1-e protein to human neurological abnormalities. Notably, there is an epidemiological association between patients with the subepidermal blistering disease, bullous pemphigoid, and an increased incidence of multiple sclerosis and also Parkinson's disease (Stinco *et al.*, 2005), as well as epilepsy, dementia, and stroke (Foureur *et al.*, 2001, 2006). Moreover, autoantibodies to BPAG1-e have been associated with various neurological disorders (Li *et al.*, 2009), and have been identified in the cerebrospinal fluid of some individuals with multiple sclerosis (Lafitte *et al.*, 2005). The *DST* mutation in our patient is also expected to disrupt BPAG1-n, although the potential significance of this is unknown, given that other studies have failed to demonstrate significant tissue expression of this isoform (Leung *et al.*, 2001b).

Whether our patient's neurological features are the consequence of either *DST* or *NOTCH3* gene pathology or from another cause is still uncertain. To the best of our knowledge, pathogenic mutations in *DST* have not been reported previously, and therefore the future identification of other individuals with additional mutations in this gene will be necessary to clarify genotype–phenotype correlation. For now, delineation of a homozygous loss-of-function mutation in the *DST* gene expands the molecular basis of inherited skin blistering and demonstrates the particular *in vivo* role of BPAG1-e in the structural organization of hemidesmosomes at the cutaneous basement membrane zone.

MATERIALS AND METHODS

Patient and biological samples

The patient provided written and informed consent according to a protocol approved by the St Thomas' Hospital Ethics Committee (molecular basis of inherited skin disease: 07/H0802/104). Blood and skin samples (ellipse of skin taken under local anesthesia using 1% lignocaine) were obtained in adherence to the Helsinki guidelines. The skin sampled was noninflamed, nonlesional skin, as, at the time of examination, the patient had no fresh blisters (i.e., <24 hours old).

Transmission electron microscopy

Skin biopsy specimens were cut into small pieces (of $<1\,mm^3$) and fixed in half-strength Karnovsky fixative for 4 hours at room temperature. After washing in 0.1 M phosphate buffer (pH 7.4), the samples were immersed in 1.3% aqueous osmium tetroxide (TAAB Laboratories, Berkshire, UK) for 2 hours, followed by incubation in 2% uranyl acetate (Bio-Rad, Hertfordshire, UK), and dehydrated in a graded ethanol series, and then embedded in epoxy resin via propylene oxide (TAAB Laboratories). Ultra-thin sections were stained with uranyl acetate and lead citrate and examined in a Philips CM10 transmission electron microscope (Philips, Eindhoven, The Netherlands).

Immunofluorescence microscopy

Skin sections measuring 5 μm were air-dried and initially blocked with diluted normal goat serum (Sigma-Aldrich, Dorset, UK), and then incubated with the following antibodies diluted in phosphate-buffered saline with 30% w/v bovine serum albumin (Sigma-Aldrich) where stated: clone BPC319 (BPAG1-e, derived from mice immunized with a recombinant human 361 amino-acid peptide within the carboxyl domain; Okumura *et al.*, 2002), used neat (source K. Owaribe); clone HD1-121 (plectin), 1:40 dilution (K. Owaribe); clones 450-9D (β4 integrin subunit) and GoH3 (α6 integrin subunit), both 1:250 dilutions (AbD Serotec, Oxford, UK); clone mAb-123 (collagen XVII), 1:50 dilution (a gift from M.P. Marinkovich, Stanford, Palo Alto, CA, USA); clone LL002 (keratin 14), 1:1,000 dilution (AbD Serotec); GB3 (laminin-332), 1:300 dilution (http://Immunologicalsdirect.com); clone LH7.2 (collagen VII), 1:1,000 dilution (Sigma-Aldrich, Poole, UK); clone COL94 (collagen IV), 1:500 dilution (Sigma-Aldrich). After washing in phosphate-buffered saline, slides were labeled with fluorescein isothiocyanate secondary antibodies (Invitrogen, Paisley, UK). Negative controls omitting the primary antibody were performed for each set of labeling experiments. All sections were photographed using the same camera and identical exposure times (3 seconds).

DNA sequencing

After obtaining informed consent, genomic DNA was extracted from a peripheral blood sample obtained from the affected

individual. For sequencing, DNA was amplified with primers sited in introns flanking individual exons of the *DST* gene (Supplementary Tables 1 and 2 online) and the *NOTCH3* gene (for further details, see Markus *et al.*, 2002). For PCR amplification, 20 ng genomic DNA was used as a template in an amplification buffer containing 6.25 pmol of primers, 5 mmol of each trinucleotide phosphate, and 0.625 Units Taq polymerase (Qiagen, Warrington, UK) in a total volume of 25 µl in a GeneAmp PCR system 9700 thermal cycler (Applied Biosystems, Crawley, UK). The amplification conditions were 94 °C for 5 minutes, followed by 40 cycles of 94 °C for 30 seconds, 60 °C for 30 seconds, and 72 °C for 45 seconds. Aliquots (5 µl) of PCR products were analyzed by electrophoresis using 3% agarose gel. PCR products were then purified using a QIAquick PCR Purification Kit (Qiagen) and sequenced directly in an ABI 3130 genetic analyzer (Applied Biosystems).

Western blotting

Primary keratinocyte cultures were established as described previously (Mee *et al.*, 2000), and maintained in a defined keratinocyte growth medium (EpiLife, Invitrogen, Renfrew, UK) for three passages. Cellular lysates were prepared from confluent 75 cm^2 flasks through addition of 1 ml preheated lysis buffer (100 mM Tris-Cl (pH 6.8), 4% SDS, 20% glycerol, 0.1% bromophenol blue, 5% β-mercaptoethanol), followed by shearing and clarification. Lysates (12 µl) were separated by denaturing SDS-PAGE using either 5% (BPAG1-e) or 7.5% (β-actin) gels, alongside 5 µl biotinylated markers (Precision Plus Protein WesternC Standards, Bio-Rad, Hemel Hempsted, UK). After transfer to nitrocellulose membranes (Protran BA 85, Whatman, Maidstone, UK), samples were blocked (1 M glycine, 1% ovalbumin, 5% dry skimmed milk, 5% fetal calf serum) for 1 hour at room temperature and probed overnight with either human anti-human BPAG1-e monoclonal antibody (5E, batch 1,748, 1:25 dilution, directed against a 114 amino-acid epitope within the carboxyl domain, source T. Hashimoto) (Hashimoto *et al.*, 1993) or rabbit anti-human β-actin polyclonal antibody (ab8227, Abcam, Cambridge, UK; 1:2,500 dilution) at 4 °C. Detection was accomplished using horseradish peroxidase-conjugated goat anti-rabbit (sc-2054) or goat anti-human (sc-2453) IgG antibody (Santa Cruz Biotechnology, Santa Cruz, CA, USA; 1:3,000 dilution) for 1 hour at room temperature and visualization was achieved using the Amersham ECL Plus Western Blotting Detection System (GE Healthcare, Slough, UK) and autoradiography.

Real-time reverse transcriptase-PCR

Total RNA obtained from patient and normal control skin was extracted using an RNeasy Fibrous Tissue Mini Kit (Qiagen). In all, 0.5 µg of total RNA was used for each 50 µl cDNA synthesis reaction with SuperScript III Reverse Transcriptase (Invitrogen), followed by ribonuclease H (Invitrogen) treatment, according to the manufacturer's protocol. Three sets of PCR primers were generated (PP1, PP2, and PP3, positions illustrated in Figure 2d). PP1 is sited upstream from the mutation (forward primer 5′-AAACGCCGAAGA ATGCAG-3′ nucleotides 3,283–3,300; reverse primer 5′-AATATGC CCCATGTTCAGAAG-3′ nucleotides 3,466–3,446; PCR product size 184 bp; GenBank NM_001724.4). PP2 spans the nonsense mutation (forward primer 5′-TGGACCTAAGGACTCGATATAC-3′ nucleotides 3,332–3,353; reverse primer 5′-TCCTCTACTCGGGACTTTTG-3′ nucleotides 3,584–3,565; PCR product size 253 bp). PP3 is sited in the 3′ untranslated region (forward primer 5′-CTTCAGAACTCCCCT TCATTG-3′ nucleotides 8,371–8,391; reverse primer 5′-GAAATGG GACATTGTGGTAAAC-3′ nucleotides 8,601–8,580; PCR product size 236 bp). The annealing temperature was 55 °C for all reactions. Real-time quantitative PCR was carried out using an ABI Prism 700 Instrument (Applied Biosystems) and SYBR Green PCR Master Mix (Applied Biosystems), following the manufacturer's recommendations. Briefly, 1 µl of cDNA solution was used in 10 µl PCR containing 1 × SYBR Green I master mix with 2.5 mM of $MgCl_2$ and primers. Each sample was run in duplicate, and each PCR run included a no-template control. The 18S ribosomal RNA expression was used as internal reference for relative quantification.

CONFLICT OF INTEREST

The authors state no conflict of interest

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SUPPLEMENTARY MATERIAL

Supplementary material is linked to the online version of the paper at http://www.nature.com/jid

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